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The Major ^{35}S-Methionine–labeled Rapidly Transported Protein (Superprotein) Is Identical to SNAP-25, a Protein of Synaptic Terminals

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Superprotein is a rapidly axonally transported protein that is conspicuously labeled with ^{35}S-methionine supplied to the cell bodies of retinal ganglion cells. Superprotein candidates are apparent among the rapidly transported proteins of many neurons from the CNS and PNS, including cranial, sympathetic, sensory, and motor neurons from mammals, fish, and amphibians. To determine the identity of Superprotein, we purified it from rabbit visual system and spinal cord and determined the amino acid sequence of seven of its tryptic peptides. The sequence shows that Superprotein is SNAP-25, a protein recently predicted from a cDNA sequence; SNAP-25 has been reported to be concentrated in the synaptic terminals of a selected population of CNS neurons. We measured the amount of radioactivity associated with Superprotein in tissue containing axons (optic tract) and synaptic terminals (superior collicules) of rabbit retinal ganglion cells. Labeled Superprotein disappeared from the superior colliculus more rapidly than another protein (synpasin I–like protein) that is concentrated in synaptic terminals. These results serve to unite the observations on the synthesis, distribution, metabolism, and axonal transport of Superprotein with observations of the primary structure and steady state distribution of SNAP-25 and its mRNA.

Proteins that are destined for axons or synaptic terminals can be labeled with radioactive amino acids supplied to the cell bodies where they are synthesized (reviewed in Willard, 1983). Electrophoretic analyses of such labeled, axonally transported proteins have indicated that different collections of proteins [designated groups I–V (Willard et al., 1974; Willard and Hulebak, 1977); groups IV and V are also designated SCb and SCa, respectively (Hoffman and Lasek, 1975)] move down the axons at different velocities, ranging from more than 240 mm/d (group I) to less than 1 mm/d (group V). The more slowly moving proteins (groups III, IV, and V) comprise cytoskeletal proteins such as tubulin, neurofilament proteins, actin, and myosin, as well as cytoplasmic enzymes, whereas group II includes mitochondrial proteins (reviewed in Grafstein and Forman, 1980; Baitinger et al., 1982). The most rapidly transported proteins (group I) are predominantly associated with membranes (e.g., Lorenz and Willard, 1978) and may represent the movement of organelles along microtubules by a mechanism involving proteins similar to kinesin (Brady, 1985; Vale et al., 1985). Although more than 150 of these radiolabeled group I proteins have been resolved by two-dimensional gel electrophoresis (Wagner et al., 1979), few have been identified. These include proteins that resemble synapsin I (De Camilli et al., 1983a,b; Huttner et al., 1983; Baitinger and Willard, 1987), the Na^+-K^+-ATPase (Specht and Sweadner, 1984) GAP-43 (Skene and Willard, 1981a,b), and the β-amyloid precursor protein (Koo et al., 1990). The nature of these identified proteins suggests that the characterization of additional group I proteins may identify proteins that are important for neuronal function.

Among the group I proteins that can be labeled in this manner with ^{35}S-methionine, one of the most conspicuous in several different neuronal systems is Superprotein, so designated to reflect its incorporation of more than 14% of the total rapidly transported ^{35}S-methionine, more than 20 times the average incorporation (Willard et al., 1974; Kelly et al., 1980; Willard, 1983). In contrast to its prominence among ^{35}S-methionine–labeled rapidly transported proteins (Fig. 1), Superprotein has not been detected by staining of electrophoretically separated proteins with Coomassie blue (Wagner et al., 1979); because of its low abundance, its characterization presents a challenging problem.

Here, we first report the kinetics of labeling of Superprotein in rabbit retinal ganglion cells and then describe the purification of seven of its tryptic peptides from rabbit nervous tissue. The sequences of these peptides show that Superprotein is identical to SNAP-25, a protein (recently predicted by a cDNA sequence) that has been reported to be concentrated in a selected population of synaptic terminals of the CNS (Oyler et al., 1989).

Materials and Methods

Labeling of axonally transported Superprotein. ^{35}S-methionine (New England Nuclear; >1000 Ci/mmol) was injected into the eye (vitreous humor) of a restrained New Zealand White rabbit; the eye had been anesthetized by topical application of proparacaine hydrochloride (0.05% solution; Rugby Laboratory, Rockville Centre, NY). After various intervals, the animals were killed by a lethal injection of sodium pentobarbital into the marginal ear vein, and the optic nerve, optic tract, lateral geniculate nucleus, and superior colliculus were removed and frozen. A particulate subcellular fraction of the tissue was prepared by homogenization in a motor-driven homogenizer (glass on glass for tissue

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containing optic nerve, Teflon on glass for other tissues) in H buffer [5 mM ethylenediaminetetraacetic acid, 5 mM dithiothreitol, 10 mM Tris, pH 8.0; phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 3 mM immediately before homogenization] followed by centrifugation at 100,000 × *g* for 1 hr. All procedures were carried out at 4°C.

Kinetics of labeling Superprotein. ^{35}S-methionine-labeled proteins from a particulate fraction of visual tissue, prepared as described above, were extracted and electrophoresed on two-dimensional gels, using nonequilibrium pH gradient gel electrophoresis in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension. The preparation of these gels has been described previously (Baitinger and Willard, 1987). After it was stained and dried, the region of the gel containing Superprotein (identified by autoradiography) was excised, hydrated for 2 hr in 0.5 ml of H_2O at 80°C, and dissolved by the addition of 2 ml of hydrogen peroxide (50%) and incubation at 80°C for 24 hr. Radioactivity was measured by scintillation counting after the addition of 10 ml of scintillation fluid (3A70, RPI, Mt. Prospect, IL). The background was determined by assessing the radioactivity in pieces of the gel of the same size taken from regions adjacent to Superprotein.

Solubility of Superprotein in various media. The initial homogenate of an optic nerve and optic tract from a rabbit whose eye had been labeled 3 hr previously was divided into seven equal parts, and particulate fractions were prepared as described above. Each pellet was homogenized in 1 ml of H buffer, containing one of the following: no additives, deoxycholate (0.1% or 1%), Triton X-100 (0.1% or 1%), urea (4 M or 8 M), SDS (1%). The homogenates were centrifuged at 100,000 × *g* for 1 hr. The resulting pellet was extracted in the same manner with 1 ml of 1% SDS in H buffer and subjected to centrifugation under the same conditions. All of the supernatants were dialyzed against a solution containing 0.01% SDS and 10^{-4} M dithiothreitol and then dried in a lyophilizer. The samples were resuspended in 0.1 ml of H buffer containing 1% SDS, heated to 95°C for 10 min, and fractionated by SDS-PAGE under conditions similar to those described by Laemmli (1970). The gel was stained with Coomassie blue, dried, and autoradiographed. The region of the gel containing Superprotein was excised and dissolved, and radioactivity was determined by liquid scintillation counting, as described above. The radioactivity extracted in the first extraction was divided by the sum of the radioactivity extracted in the two sequential extractions of each pellet to give the fraction of total extractable Superprotein that was soluble in the first extracting reagent.

Purification of radiolabeled Superprotein from visual tissue and spinal cord. A particulate fraction of both optic nerves, optic tracts, superior colliculi, and lateral geniculates, from an adult rabbit killed 4 hr after 2 mCi of ^{35}S-methionine was injected into each eye, was prepared as described above. The tissue (0.7 gm) was homogenized in 7 ml of H buffer (with PMSF added immediately before homogenization) and then diluted to 20 ml before centrifugation. The resulting particulate fraction was homogenized in H buffer (9 ml) containing SDS (1.5%) and then heated to 90°C for 10 min. After centrifugation at 100,000 × *g* for 1 hr at 15°C the supernatant was subjected to SDS-PAGE on three single-well slab gels (separating gel was 12.5 cm high, 15 cm wide, 3 mm thick; stacking gel was 2 cm high) under conditions similar to those described by Laemmli (1970). The separating gel was composed of acrylamide (10%) and bisacrylamide (0.3%), and the stacking gel contained acrylamide (5%) and bisacrylamide (0.15%). After electrophoresis (monitored by the migration of a trace of bromphenol blue dye that had been added to the sample), the gels were stained with Coomassie brilliant blue R (0.25%) in an aqueous solution of methanol (40%) and acetic acid (10%) for 1 hr and then destained for 20 hr in an aqueous solution of methanol (25%) and acetic acid (10%). Initially, a vertical strip of such a gel was dried and autoradiographed to determine the migration of Superprotein relative to the stained bands. Subsequently, these stained marker proteins were used as references to excise a longitudinal strip containing Superprotein. The strip was cut into pieces (5 cm^3) and washed three to five times for 10 min in a solution containing Tris (10 mM, pH 8.0) until the pH of the wash solution was 8.0. After their volume was measured by H_2O displacement, the pieces were ground with a mortar and pestle and then agitated for 20 hr at room temperature in H buffer (2× their volume) containing 1.5% SDS. The extraction medium was separated from the gels by filtration through glass wool fitted in a plastic syringe. The proteins were precipitated and separated from SDS by mixing the extract with an equal volume of acetone, prechilled to −20°C, after incubation for 1 hr at −20°C, the proteins were collected by centrifugation at 14,000 × *g* for 15 min at 4°C. The pellet was washed twice with the same volume of acetone at −20°C, dried, and resuspended in 1.5 ml of H buffer containing 1.5% SDS. After addition of 0.15 vol of glycerol and a trace of bromphenol blue dye, the solution was heated at 90°C for 5 min and electrophoresed on a single 1-mm-thick slab gel of the same size and composition as described above, except that the separating gel contained 8 M urea. The strip containing Superprotein was identified, extracted from the gel, and separated from SDS by acetone precipitation exactly as described below.

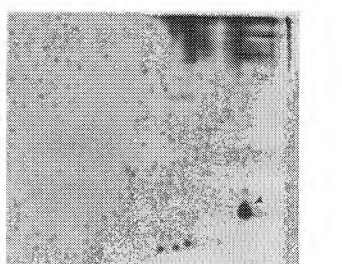

Figure 1. Typical appearance of Superprotein (*arrowhead*) on an autoradiograph of a two-dimensional gel. Proteins from a particulate subcellular fraction of the optic tract of a rabbit whose eye had been labeled for 3 hr with ^{35}S-methionine were fractionated by two-dimensional gel electrophoresis using nonequilibrium pH gradient gel electrophoresis in the first dimension (acidic end is at the right) and SDS-PAGE in the second dimension. The figure shows an autoradiograph of this gel. Modified from Baitinger and Willard (1987). The appearance of Superprotein on autoradiographs of isoelectric focusing SDS-PAGE two-dimensional gels is illustrated in Doster et al. (1991).

Superprotein (unlabeled) was purified from 2 gm of rabbit spinal cord by the same procedure, except that five slab gels were used for the initial electrophoresis (in the absence of urea). After the first electrophoretic step, the nature of the protein extracted from the longitudinal gel strip was verified by comparing its electrophoretic mobility on minigels (6 cm high, 10% acrylamide) with both the proteins extracted from adjacent strips on either side of the presumptive Superprotein strip as well as with labeled Superprotein purified from the visual system (detected by autoradiography of the minigel). Purified labeled Superprotein (335,000 cpm) was added to the preparation prior to the second (8 M urea) electrophoretic step. The comigration of the spinal cord protein and Superprotein on this gel was confirmed initially by scintillation counting of the extract (as well as extracts of adjacent gel strips) and then verified by comparison of the stained band and labeled marker after SDS-PAGE on minigels in the presence and absence of 8 M urea.

Analysis of Superprotein peptides. The final acetone precipitate of spinal cord Superprotein preparation mixed with labeled visual system Superprotein (91,000 cpm was recovered) was suspended in 50 μl of H buffer lacking EDTA and containing 0.005% SDS, and incubated with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma Chemical Company, St. Louis, MO) at a ratio of 1:25 (trypsin : Superprotein; the concentration of Superprotein was roughly estimated from its intensity of staining with Coomassie blue on analytical SDS gels) for 18 hr at 37°C. The reaction was terminated by heating in boiling H_2O for 5 min, and insoluble material was removed by centrifugation in an Airfuge (Beckman, Palo Alto, CA) for 10 min.

The peptides were separated by reversed-phase high-pressure liquid chromatography (HPLC) on a C18 column (#218TP54, Vydac, Hesperia, CA) using a Waters Millipore (Milford, MA) HPLC system. After dilution to 2 ml with buffer A (10 mM Na_2HPO_4, pH 6.9), the sample

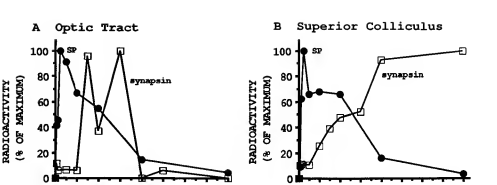

Figure 2. Time course of labeling of Superprotein in an axon-containing structure (*Optic Tract*) and synaptic terminal–containing structure (*Superior Colliculus*). The radioactivity associated with Superprotein at the indicated times after rabbits' eyes were labeled with ^{35}S-methionine was determined by scintillation counting of Superprotein excised from gels such as that shown in Figure 1. The time course of labeling of proteins that resemble synapsin I, a synaptic vesicle–associated protein, determined by densitometric scanning of these same gels [previously reported in Baitinger and Willard (1987)] is shown for comparison.

was applied and the column was washed for 5 min in buffer A at a rate of 1 ml/min. The peptides were eluted with a gradient of 0.25%/min buffer B (70% acetonitrile, 5 mM NA_2HPO_4, pH 6.9) for 20 min, followed by 0.6%/min buffer B for 50 min. The optical density was monitored by absorption at 214 nm, and the radioactivity in the 1 ml fractions was monitored by scintillation counting of an aliquot. Fractions containing radioactive peptides were concentrated by evaporation and chromatographed at lower pH (pH ≈ 2) on a second C18 column, equilibrated with buffer A′ (0.1% trifluoroacetic acid). The column was developed with a gradient of 0.15%/min buffer B′ (0.1% trifluoroacetic acid, 95% acetonitrile) at 1 ml/min beginning at a concentration of acetonitrile that was 15% below the concentration at which the peptide had been eluted from the first column, and ending after a 30% change in acetonitrile concentration.

Fractions containing radioactivity were immobilized on a glass fiber support and subjected to amino acid sequence analysis by automated sequential Edman degradation, using an Applied Biosystems (Foster City, CA) model 470A protein sequencer and the programs recommended by the manufacturer. After each cycle of degradation, approximately 40% of the sample containing the phenylisothiocyanate derivative removed from the protein was used to determine whether it contained ^{35}S-methionine by liquid scintillation counting; the remainder was used for amino acid analysis.

Relationship between steady state distribution, transport velocity, and half-life of an axonally transported protein. To understand how the axonal transport velocity and rate of turnover of a protein might be related to its steady state distribution between axons and synaptic terminals revealed by antibody staining, we considered a protein that is transported at velocity v, through an axon of cross-sectional area A, to a destination compartment of volume V_a where its turnover can be described by the first-order rate constant k. The rate of arrival of protein in the destination compartment is vAC_o, where C_o is the concentration of the protein in the axon. The rate of disappearance of the protein from the destination compartment is kC_iV_i, where C_i is the concentration in the destination compartment and V_i is the volume of this compartment. Because the decay of the protein is assumed to be first order, $k = (\ln 2)/T_{1/2}$, where $T_{1/2}$ is the half-life of the protein in the destination compartment. At steady state, $vAC_o = (\ln 2)C_iV_i/T_{1/2}$, or, expressed as the steady state quotient of the concentration in the destination compartment and the concentration in the transport compartment,

$$\frac{C_i}{C_a} = \frac{1.4vAT_{1/2}}{V_i}.$$

Results

Kinetics of labeling of Superprotein in axons and terminals of rabbit retinal ganglion cells

In previous experiments to study the axonal transport of proteins resembling synapsin I (Baitinger and Willard, 1987), ^{35}S-methionine (0.5 mCi) was injected into the vitreous of adult rabbits at various times prior to death (3 hr, 6 hr, 12 hr, 1 d, 2 d, 4 d, 8 d, and 16 d). Proteins from a particulate subcellular fraction of the tissues containing the axons (optic nerve and optic tract) and synaptic terminals (LGN and superior colliculus) of the retinal ganglion cells were extracted and electrophoresed on two-dimensional gels that employed nonequilibrium pH gradient electrophoresis in the first dimension and SDS-PAGE in the second dimension. Figure 1 shows an autoradiograph of one such gel; additional examples are shown in Baitinger and Willard (1987) (see their Figs. 3, 4). To measure the radioactivity associated with Superprotein, we excised the appropriate spot from these same gels and determined the radioactivity by liquid scintillation counting as described in Materials and Methods. Figure 2 shows the time course of labeling of Superprotein in tissue containing axons (optic tract, Fig. 2*A*) and synaptic terminals (superior colliculus, Fig. 2*B*). In both tissues, Superprotein-associated radioactivity was detected by 3 hr after isotope injection, reached maximum levels by 12 hr, and declined more than 90% by 8 d after labeling. A comparison of the time course of labeling of Superprotein with synapsin I–like proteins (previously reported; Baitinger and Willard, 1987) shows that both proteins arrive in the optic tract by 3 hr after their synthesis; on the other hand, the maximum labeling of Superprotein (12 hr) precedes synapsin I (2–6 d), suggesting that a major portion of synapsin I–like protein is transported more slowly than Superprotein. Most of the label associated with both proteins had disappeared from the optic tract by 8 d after synthesis. In the superior colliculus, the synapsin I–like protein remained labeled much longer than Superprotein, reaching maximum levels at 16 d, a time when the label associated with Superprotein was negligible (Fig. 2*B*).

Solubility of Superprotein

Prior to purifying Superprotein, we investigated its extraction by several agents as described in Materials and Methods. These experiments showed that more than half of the Superprotein that could be extracted by 1% SDS was extracted by 1% deoxycholate, 0.1% or 1% Triton X-100, or 8 M urea; however, extraction by 1% SDS was most efficient. On the other hand, 0.1% deoxycholate and 4 M urea were ineffective solubilizing agents.

Purification and sequencing of tryptic peptides from Superprotein

To purify Superprotein, we made use of an unusual sensitivity to urea of its mobility on SDS gels, illustrated in Figure 3. Lanes 5 are autoradiographs of ^{35}S-methionine–labeled rapidly trans-

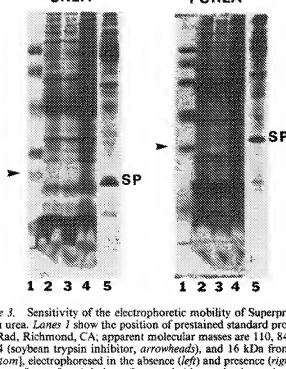

Figure 3. Sensitivity of the electrophoretic mobility of Superprotein to 8 M urea. *Lanes 1* show the position of prestained standard proteins [Bio-Rad, Richmond, CA; apparent molecular masses are 110, 84, 47, 33, 24 (soybean trypsin inhibitor, *arrowheads*), and 16 kDa from *top to bottom*], electrophoresed in the absence (*left*) and presence (*right*) of 8 M urea. *Lanes 2* and *3* show Coomassie blue–stained proteins in a crude particulate subcellular fraction of spinal cord that served as the starting material for Superprotein purifications. *Lanes 4* show the Coomassie blue–stained proteins from a similar particulate fraction of combined optic nerve, optic tract, LGN, and superior colliculus from a rabbit whose eye had been labeled for 4 hr with ^{35}S-methionine. *Lanes 5* show an autoradiograph of *lanes 4*, revealing the position of radiolabeled Superprotein (*SP*). Note its change in position relative to soybean trypsin inhibitor (*arrowheads*) in the presence and absence of 8 M urea.

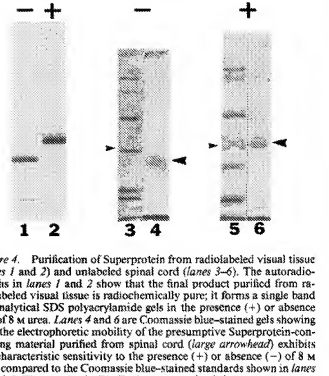

Figure 4. Purification of Superprotein from radiolabeled visual tissue (*lanes 1* and *2*) and unlabeled spinal cord (*lanes 3–6*). The autoradiographs in *lanes 1* and *2* show that the final product purified from radiolabeled visual tissue is radiochemically pure; it forms a single band on analytical SDS polyacrylamide gels in the presence (+) or absence (–) of 8 M urea. *Lanes 4* and *6* are Coomassie blue–stained gels showing that the electrophoretic mobility of the presumptive Superprotein-containing material purified from spinal cord (*large arrowhead*) exhibits the characteristic sensitivity to the presence (+) or absence (–) of 8 M urea compared to the Coomassie blue–stained standards shown in *lanes 3* and *5*. The standards (Bio-Rad, not prestained) have apparent molecular masses of 97, 66, 45, 21 (soybean trypsin inhibitor, *small arrowheads*), and 16 kDa from *top to bottom*.

ported proteins separated by SDS-PAGE in the presence (+ urea) or absence (– urea) of 8 M urea. The mobility of Superprotein (SP) is retarded by urea relative to standards (lanes 1) and relative to stained proteins from rabbit spinal cord (lanes 2 and 3) or visual system (lanes 4) that were electrophoresed on the same gels.

We first purified ^{35}S-methionine–labeled axonally transported Superprotein from the optic nerves, tracts, lateral geniculate nuclei, and superior colliculi of a rabbit whose retinas had been labeled with ^{35}S-methionine 4 hr previously. After electrophoresis in the absence of urea, proteins were extracted from the region of the gel that contained Superprotein, and reelectrophoresed in the presence of urea, as described in Materials and Methods. This served to separate Superprotein from other proteins with which it initially coelectrophoresed. The resulting preparation was radiochemically pure; it produced a single autoradiographic band of the appropriate mobility in the presence or absence of 8 M urea (Fig. 4, lanes 1, 2).

This radiochemically pure preparation of Superprotein was used as a marker to fractionate larger amounts of Superprotein from a particulate fraction of unlabeled rabbit spinal cord by the same sequence of procedures as described above and in Materials and Methods. The resulting material formed a broad electrophoretic band whose mobility relative to standards was sensitive to urea (Fig. 4, lanes 3–6). Attempts to determine the amino-terminal amino acid sequence of this material were unsuccessful, suggesting that the amino terminus was blocked.

After it was separated from SDS by means of acetone precipitation, the mixture of unlabeled protein from spinal cord and radiolabeled, axonally transported Superprotein purified from visual system was resuspended and digested with trypsin. The resulting tryptic peptides were separated by reversed-phase chromatography on a C18 matrix at pH 6.9. Figure 5 (upper half) shows that more than 12 peaks of radioactivity were separated; the amount of material associated with these radioactive peaks was insufficient to generate distinct optical density peaks (Fig. 5, lower half). Initial attempts to determine the amino acid sequence of the material in these fractions indicated that some contained multiple peptides. We therefore rechromatographed each of the 12 fractions numbered in Figure 5 in a medium of lower pH (≈2). Figure 6 illustrates that this procedure effectively separated multiple peptides contained in certain fractions (e.g., peak 3). The material in fractions containing radioactivity was subjected to amino-terminal sequence analysis by automated sequential Edman degradation. A single sequence was generated by the material in each of seven fractions (Table 1). In one case (peak 3B), no phenylthiohydantoin derivatives of amino acids were detected, and approximately 80% of the initial radioactivity remained associated with the glass fiber support after five sequencing cycles. It is therefore likely that this peak contains a peptide from the blocked N-terminus of the protein. In the case of peaks 1 and 3, the sequences were identical. Most likely, the methionine in peak 1 was oxidized, forming methionine sulfoxide and altering its mobility on the reversed-phase column.

All of the sequences met three criteria for derivation from Superprotein. First, each contains a methionine residue, as does

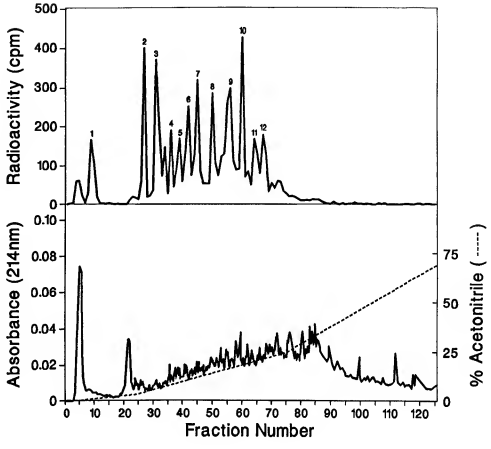

Figure 5. Elution of tryptic peptides of the Superprotein preparation from the first (pH 6.9) HPLC C18 reversed-phase column. The *upper half* shows the elution of ^{35}S-containing material (*peaks 1–12*), determined by scintillation counting. The *lower half* shows the ultraviolet light absorption monitored at 214 nm.

the Superprotein peptide in the same fraction (because ^{35}S-methionine from Superprotein that had been axonally transported *in vivo* was the source of the radioactivity in the fraction). A second, more stringent criterion is that ^{35}S-radioactivity derived from the marker Superpeptide, and the methionine detected by sequence analysis, should be located at the same position relative to the amino terminus. To test this, a portion of the material released after each cycle of Edman degradation was assayed for ^{35}S-radioactivity. Figure 7 shows that the major release of radioactivity and methionine coincided in each peptide. Third, the specific activity of the methionine, estimated from the released radioactivity and the absorbance of the phenylthiohydantoin derivative of methionine, was similar for all of the peptides, as would be expected if they were all derived from the

Table 1. Tryptic peptides of Superprotein

Peptide[a]	Elution, pH 6.9 (% acetonitrile)	Elution, pH 2 (% acetonitrile)	^{35}S recovered from pH 2 column (%)[b]	Sequence[c]	^{35}S recovered at methionine cycle (%)[d]	Specific activity (cpm/pmol)
1	0.80	3.49	27	IMEK	103	16
2	3.85	3.92	82	MLGSG	70	24
3A	3.92	5.56	28	IMEK	59	28
3B	3.92	12.83	41	No sequence	0	–
8	11.9	18.15	28	XLVMLDEQGEQLER	24	23
9	14.9	18.0	30	MLQLVEESSKDAGIR	47	16
10A	16.10	17.20	21	EQMAI	45	24
10B	16.10	19.23	33	VVDEREQMAISGGFIR	16	23

[a] Peptides are designated by number according to their elution from a C18 column at pH 6.9 (Fig. 5) and by letter according to their elution from a second C18 column at pH 2 (e.g., Fig. 6).

[b] Percentage of radioactivity applied to the column.

[c] X indicates an unidentified amino acid.

[d] Percentage of radioactivity applied to glass fiber sequencing support.

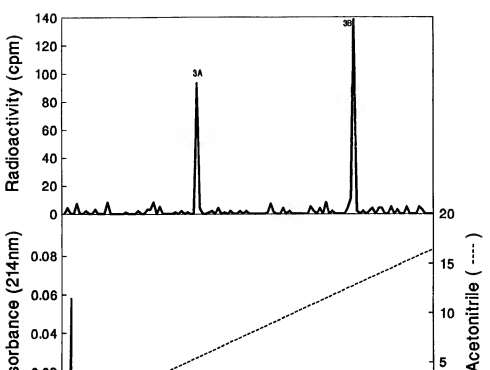

Figure 6. Elution of presumptive Superprotein tryptic peptides from the second (pH ≃ 2) C18 reversed-phase column. The fraction from the first column (Fig. 5) containing peak 3 was rechromatographed at lower pH, revealing that this material contained two radioactive peptides, *3A* and *3B* (*upper half*). The *lower half* of the figure illustrates that these peaks contained little ultraviolet light–absorbing material (monitored at 214 nm). Each of the fractions in Figure 5 that contained numbered peaks of radioactivity was rechromatographed in this manner.

same protein (Table 1). These observations show that the sequence represents methionine-containing peptides of Superprotein.

To determine whether the sequence of Superprotein is novel or corresponds to a previously described sequence, the EMBL data base was searched for similar sequences. This revealed that the exact sequences of all of these Superprotein peptides are contained in the sequence of a protein designated SNAP-25, recently deduced from the sequence of a rat cDNA. No other sequences in the data base matched the Superprotein sequences. The location of these peptides (which comprise 25% of the sequence of SNAP-25) within the sequence of rat SNAP-25 is illustrated in Figure 8.

Discussion

The experiments reported here show that Superprotein is identical to the protein SNAP-25. We first utilized the rapid transport process to separate labeled Superprotein from other labeled retinal proteins, and further separated Superprotein from other group I rapidly transported proteins by SDS-PAGE, exploiting a differential shift in its electrophoretic mobility caused by urea. Using this radiochemically pure Superprotein as a marker, we purified unlabeled Superprotein from spinal cord tissue by these same procedures. Radioactive tryptic peptides isolated from this material by two cycles of reversed-phase chromatography produced single amino acid sequences. Although the sequences most likely reflected the more abundant material from spinal cord, their derivation from Superprotein was confirmed by the coincidence of release of radioactivity from the labeled marker Superpeptide with the release of methionine. The sequences of these peptides exactly matched the sequence of SNAP-25 and comprise 25% of its amino acids. This general strategy may prove useful in the identification of additional axonally transported proteins.

The identity of Superprotein and SNAP-25 serves to unite the previously determined characteristics of each. Superprotein was first described as a major ^{35}S-methionine–labeled rapidly axonally transported protein in rabbits (Willard et al., 1974; designated protein 20); Superprotein candidates can be identified in many publications (Table 2) by virtue of their prodigious incorporation of ^{35}S-methionine and their distinctive electrophoretic mobilities. SNAP-25 was initially described as a cDNA that preferentially hybridized with RNA from brain (compared to liver); this RNA was fourfold more abundant in the CA3 region than in the CA1 region of the hippocampus (Branks and Wilson, 1986; Oyler et al., 1989). The predicted sequence of SNAP-25 contains 13 methionine residues (6%) and two proline residues (1%), which may account for the efficient labeling of Superprotein with ^{35}S-methionine relative to other transported proteins and relative to its labeling with proline (Estridge and Bunge, 1978; Willard, 1983, his Fig. 3). Subcellular fractionation experiments have indicated that both Superprotein (Lorenz and Willard, 1978) and SNAP-25 (Oyler et al., 1989) are intimately associated with material resembling the plasma membrane. The absence in the sequence of SNAP-25 of a hydrophobic region of sufficient length to span the plasma membrane has suggested that its association with the plasma membrane may be mediated by other mechanisms, such as an amphipathic helix at the amino-terminal end, or associated fatty acids (Oyler et al., 1989). Although the sequence of SNAP-25 contains several potential

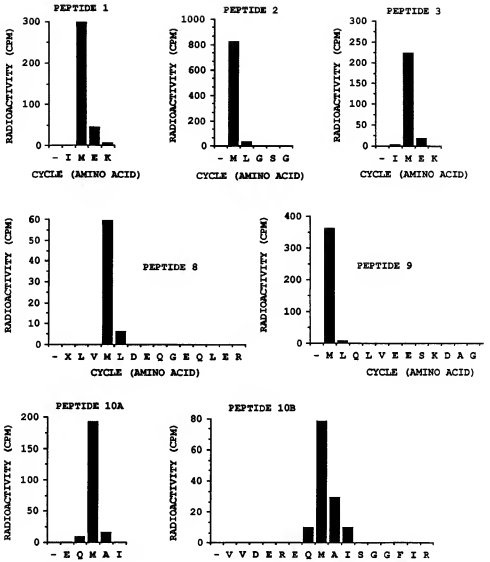

Figure 7. Corelease of ^{35}S and methionine from the solid support during sequential Edman degradation. Fractions containing radioactive peptides are designated according to the numbered peaks in Figure 5. After each cycle of Edman degradation, 60% of the material released from the solid support was subjected to amino acid analysis (*abscissa*), and 40% to liquid scintillation counting (*ordinate*). In each peptide, the release of ^{35}S-radioactivity occurred at the same cycle as the release of the phenylthiohydantoin derivative of methionine, showing that the sequenced peptide and the radioactive peptide were the same.

sites for phosphorylation and glycosylation (Oyler et al., 1989), incorporation of $^{32}PO_4$ into Superprotein has not been observed in cultured superior cervical ganglion cells (evident in Meiri et al., 1988), nor has the incorporation of radiolabeled fucose into Superprotein been detected, suggesting that it is not a fucosylated glycoprotein (Tytell et al., 1980; also evident in Skene and Willard, 1981c). A previous analysis of the ^{35}S-methionine–labeled peptides of a Superprotein candidate by thin-layer chromatography suggested a resemblance to calmodulin (Tashiro et al., 1980). However, the sequence of calmodulin and SNAP-25 appears dissimilar, suggesting either that this Superprotein candidate was in fact a different protein or that the comigration of its peptides with calmodulin was fortuitous. During development, the rate of synthesis of Superprotein is reported to increase 50–100-fold in retinal ganglion cells between the second postnatal day and adulthood in rats (Snipes et al. 1987; designated 29K) and similarly in hamsters (Moya et al., 1987; designated 27K). These developmental studies suggest that its function may

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                                    9          8
MAEDADMRNELEEMQRRADQLADESLEWTRFMLQLVEESKDAGIRTLVMLDEQGEQLER 59

IEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNKLKSSDAYKKAWGNNQDGVVASQPA 118

      10B,A
RVVDERFQMAISGGFIRRVTNDARENEMDENLEEVSGIIGNLRHMALDMGNEIDTQNRQ 177

  1,3                 2
IDRIMEKADSNKTRIDEANQRATKMLGSG                                 206
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Figure 8. Location of the sequenced Superprotein tryptic peptides within the sequence of SNAP-25 (Oyler et al., 1989). Tryptic peptide sequences, shown in *boldface*, are designated according to the numbered peaks in Figure 5.

Table 2. Superprotein candidates

Neuron	Species	Criteria[a]	Reference(s) (designation)[b]
Retinal ganglion cells	Rabbit	1D, 2D, M, U	Willard et al., 1974 (20) Wagner et al., 1979 Kelly et al., 1980 (A) Lorenz and Willard, 1978 (20) Willard, 1983
	Guinea pig	1D, 2D	Levine and Willard, 1980 Tytell et al., 1981 (23K)
	Rat	1D, 2D	Freeman et al., 1986 (29K) Snipes et al., 1987 Doster et al., 1991
	Hamster	2D	Moya et al., 1987
	Toad	1D, 2D, U	Skene and Willard, 1981a
	Goldfish	1D, 2D	Freeman et al., 1986 (29K) Benowitz and Lewis, 1983
Geniculocortical neurons	Rat	1D	Padilla et al., 1979
Spiral ganglion neurons	Rat	1D, 2D, U	Tytell et al., 1980
Nigrostriatal neurons	Rat	1D	Padilla et al., 1979
Vagus nerve	Rabbit	1D, 2D, U	Skene and Willard, 1981b
	Guinea pig	1D, 2D	Tashiro et al., 1980
Hypoglossal nerve	Rabbit	1D, 2D, U	Skene and Willard, 1981b
Ventral horn motor neurons	Rat	1D	Bisby, 1981 (S1) Oka and Brimijoin, 1990
Dorsal root ganglion cells	Rat	2D	Perry and Wilson, 1983 (C1) Nealeet et al., 1980
	Frog	2D	Hammerschlag et al., 1982 Stone et al., 1978 Perry et al., 1983 (C1) Perry et al., 1987 (C1) Stone et al., 1984 Perry and Wilson, 1983 (C1) Stone and Wilson, 1978 (C1)
Superior cervical ganglion	Rat	1D, M	Estridge and Bunge, 1978 (E3)

[a] All Superprotein candidates were intensely labeled with ^{35}S-methionine and underwent rapid axonal transport. In addition, the following characteristics were shared with Superprotein as indicated: 1D, electrophoretic mobility on one-dimensional SDS polyacrylamide gels; 2D, electrophoretic mobility on two-dimensional gels; M, specific labeling with methionines versus proline (or leucine); U, sensitivity of electrophoretic mobility to 8 M urea.
[b] The designation of Superprotein in other publications is indicated in parentheses.

be most relevant to mature neurons, consistent with the suggestion that SNAP-25 may perform a role in synaptic transmission (Oyler et al., 1989). The biosynthetic labeling of Superprotein with ^{35}S-methionine has been reported to be relatively unresponsive to axon injury in rabbit (Skene and Willard, 1981b), rat (Freeman et al., 1986), or toad (Skene and Willard, 1981a) retinal ganglion cells, frog dorsal root ganglion cells (Perry et al., 1983, 1987), and rat corticospinal neurons injured in the medulla at the level of the pyramids (Reh et al., 1987).

In situ hybridization studies of the distribution of SNAP-25 RNA have indicated that it is relatively enriched in neurons of the neocortex, hippocampus, piriform cortex, anterior thalamic nuclei, and pontine nuclei, and in granule cells of the cerebellum (Oyler et al., 1989). The basal ganglia and hypothalamus (with the exception of the arcuate nucleus) had low levels of SNAP-25 RNA. Antibodies against SNAP-25 are reported to label preferentially the synaptic terminals of a subpopulation of CNS neurons; these include the CA3 mossy fiber terminal field of the hippocampus, and the molecular layer of the dentate gyrus. Studies of Superprotein suggest that this protein is, in addition, an important component of the rapidly axonally transported proteins in retinal ganglion cells and geniculocortical, auditory, superior cervical ganglion, hypoglossal, vagus, and spinal motor neurons (Table 2). Differences in the labeling of Superprotein relative to other rapidly transported proteins in these neurons (e.g., low labeling in the hypoglossal nerve, evident in Skene and Willard, 1981b) suggest that it may be differentially expressed in these systems.

Both electron microscopy of antibody-labeled tissue and subcellular fractionation experiments have indicated that SNAP-25 is concentrated specifically in presynaptic membranes, suggesting that SNAP-25 performs a synaptic function (Oyler et al., 1989). In view of this evidence for a synapse-specific steady state distribution of SNAP-25, it is interesting that the spatial–temporal pattern of labeling of Superprotein with ^{35}S-methionine in the visual system differs from that of proteins resembling synapsin I, another protein that is concentrated in synaptic terminals (De Camilli et al., 1983a,b; Navone et al., 1984) and is considered to play an important role in synaptic vesicle function (Huttner et al., 1983; Krebs et al., 1986). Although both labeled proteins had disappeared from the axons of the optic tract by 8 d after they were synthesized, labeled synapsin I–like proteins

persisted in the superior colliculus (which contains axon terminals) for much longer than Superprotein (Fig. 2). A partial explanation for this difference is that a major fraction of the labeled synapsin I moves more slowly than Superprotein (Baitinger and Willard, 1987) and arrives in the superior colliculus later than Superprotein. Another factor that could potentially be related to this difference is the size of the terminal compartment that is occupied by each protein. Under certain conditions (see Materials and Methods) two terminal-specific transported proteins might have the same steady state distribution between terminals and axons if the protein (e.g., Superprotein) that occupied the smaller compartment (e.g., the surface of the presynaptic membrane) had a shorter half-life than the protein (e.g., synapsin I) that occupied the larger compartment (e.g., the surface of vesicles filling a portion of the synaptic terminal). This relationship is derived in Materials and Methods. It may also be relevant to the synapse-specific distribution of SNAP-25 that a Superprotein candidate designated S1 has been observed among the labeled rapidly transported proteins that are returned to the cell body by retrograde transport in rat motor neurons (Bisby, 1981). It is reported that the label associated with this protein is specifically depleted relative to the other retrogradely moving proteins (Bisby, 1981), consistent with the possibility that it is specifically retained at the axon terminal.

References

Baitinger C, Levine J, Lorenz T, Simon C, Skene P, Willard M (1982) Characteristics of axonally transported proteins. In: Axoplasmic transport (Weiss DG, ed) pp 110–120. Berlin, Heidelberg: Springer-Verlag.

Baitinger C, Willard M (1987) Axonal transport of synapsin I–like proteins in rabbit retinal ganglion cells. J Neurosci 7:3723–3735.

Benowitz LI, Lewis ER (1983) Increased transport of 44,000- to 49,000-dalton acidic proteins during regeneration of the goldfish optic nerve: a two-dimensional gel analysis. J Neurosci 3:2153–2163.

Bisby MA (1981) Reversal of axonal transport: similarity of proteins transported in anterograde and retrograde directions. J Neurochem 36:741–745.

Brady ST (1985) A novel brain ATPase with properties expected for the fast axonal transport motor. Nature 317:73–75.

Branks PL, Wilson MC (1986) Patterns of gene expression in murine brain revealed by *in situ* hybridization of brain specific mRNAs. Mol Brain Res 1:1–16.

De Camilli P, Cameron R, Greengard P (1983a) Synapsin I (protein I), a nerve terminal–specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. J Cell Biol 96:1337–1354.

De Camilli P, Harris SM Jr, Huttner WB, Greengard P (1983b) Synapsin I (protein I), a nerve terminal–specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. J Cell Biol 96:1355–1373.

Doster SK, Lozano AM, Aguayo AJ, Willard MB (1991) Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. Neuron 6:635–647.

Estridge M, Bunge R (1978) Compositional analysis of growing axons from rat sympathetic neurons. J Cell Biol 79:138–155.

Freeman JA, Bock S, Deaton M, McGuire B, Norden JJ, Snipes GJ (1986) Axonal and glial proteins associated with development and response to injury to the rat and goldfish optic nerve. Exp Brain Res 13:34–47.

Grafstein B, Forman DS (1980) Intracellular transport in neurons. Physiol Rev 60:1167–1283.

Hammerschlag R, Stone GC, Bolen FA, Lindsey JD, Ellisman MH (1982) Evidence that all newly synthesized proteins destined for fast axonal transport pass through the Golgi apparatus. J Cell Biol 93:568–575.

Hoffman PF, Lasek RJ (1975) The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons. J Cell Biol 66:351–366.

Huttner WB, Schiebler W, Greengard P, De Camilli P (1983) Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. J Cell Biol 96:1374–1388.

Kelly A, Wagner JA, Kelly RB (1980) Properties of individual nerve terminal proteins identified by two-dimensional gel electrophoresis. Brain Res 185:192–197.

Koo EH, Sisodia SS, Archer DR, Martin LJ, Weidemann A, Beyreuther K, Fischer P, Masters CL, Price DL (1990) Precursor of amyloid protein in Alzheimer disease undergocs fast anterograde axonal transport. Proc Natl Acad Sci USA 65:1561–1565.

Krebs KE, Zagon IS, Goodman SR (1986) Synapsin I stimulates the spectrin (240/235)/F-actin interaction in a phosphorylation-dependent manner. J Cell Biol 103:272a.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Levine J, Willard B (1980) The composition and organization of axonally transported proteins in the retinal ganglion cells of the guinea pig. Brain Res 194:137–154.

Lorenz T, Willard M (1978) Subcellular distribution of intraaxonally transported polypeptides in the rabbit visual system. Proc Natl Acad Sci USA 75:505–509.

Meiri KF, Willard M, Johnson MI (1988) Distribution of phosphorylation of the growth-associated protein GAP-43 in regenerating sympathetic neurons in culture. J Neurosci 8:2571–2581.

Moya KL, Benowitz LI, Jhaveri S, Schneider GE (1987) Enhanced visualization of axonally transported proteins in the immature CNS by suppression of systemic labeling. Dev Brain Res 31:183–191.

Navone F, Greengard P, De Camilli P (1984) Synapsin I in nerve terminals: selective association with small synaptic vesicles. Science 226:1209–1211.

Neale JH, Forman DS, Shibla DB, Shortell SA (1980) Comparative analysis of rapidly transported axonal proteins in sensory neurons of the frog and rat. J Neurochem 35:838–843.

Oka N, Brimijoin S (1990) Premature onset of fast axonal transport in bromophenylacetylurea neuropathy: an electrophoretic analysis of proteins exported into motor nerve. Brain Res 509:107–110.

Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE, Wilson MC (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J Cell Biol 109:3039–3052.

Padilla SS, Roger LJ, Toews AD, Goodrum JF, Morell P (1979) Comparison of proteins transported in different tracts of the central nervous system. Brain Res 176:407–411.

Perry GW, Wilson DL (1981) Protein synthesis and axonal transport during nerve regeneration. J Neurochem 37:1203–1217.

Perry GW, Wilson DL (1983) Polypeptides in frog and rat: evolutionary changes in rapidly transported and abundant nerve proteins. J Neurochem 41:772–779.

Perry GW, Krayanek SR, Wilson DL (1983) Protein synthesis and rapid axonal transport during regrowth of dorsal root axons. J Neurochem 40:1590–1597.

Perry GW, Krayanek SR, Wilson DL (1987) Effects of a conditioning lesion on bullfrog sciatic nerve regeneration: analysis of fast axonally transported proteins. Brain Res 423:1–12.

Reh TA, Redshaw JD, Bisby MA (1987) Axons of the pyramidal tract do not increase their transport of growth-associated proteins after axotomy. Mol Brain Res 2:1–6.

Skene JHP, Willard MB (1981a) Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. J Cell Biol 89:86–95.

Skene JHP, Willard MB (1981b) Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous systems. J Cell Biol 89:96–103.

Skene JHP, Willard MB (1981c) Characteristics of growth-associated polypeptides in regenerating toad retinal ganglion cell axons. J Neurosci 1:419–426.

Snipes GJ, Costello B, McGuire CB, Mayes BN, Bock SS, Norden JJ, Freeman JA (1987) Regulation of specific neuronal and nonneuronal proteins during development and following injury in the rat central nervous system. Prog Brain Res 71:155–175.

Stone GC, Wilson DL, Hall ME (1978) Two-dimensional gel electrophoresis of proteins in rapid axoplasmic transport. Brain Res 144: 287–302.

Stone GC, Hammerschlag R, Bobinski JA (1984) Involvement of coated vesicles in the initiation of fast axonal transport. Brain Res 291:219–228.

Tashiro T, Kasai H, Kurokawa M (1980) A calmodulin-related polypeptide rapidly migrates within the mammalian nerve. Biomed Res 1:292–299.

Tytell M, Gulley RL, Wenthold RJ, Lasek RJ (1980) Fast axonal transport in auditory neurons of the guinea pig: a rapidly turned-over glycoprotein. Proc Natl Acad Sci USA 77:3042–3046.

Tytell M, Black MM, Garner JA, Lasek RJ (1981) Axonal transport: each major rate component reflects the movement of distinct macromolecular complexes. Science 214:179–181.

Vale RD, Reese TS, Sheetz MP (1985) Identification of a novel force generating protein (kinesin) involved in microtubule-based motility. Cell 41:39–50.

Wagner JA, Schick-Kelly A, Kelly RB (1979) Nerve terminal proteins of the rabbit visual relay nuclei identified by axonal transport and two-dimensional gel electrophoresis. Brain Res 168:97–117.

Willard M, Cowan WM, Vagelos PR (1974) The polypeptide composition of intra-axonally transported proteins: evidence for four transport velocities. Proc Natl Acad Sci USA 71:2183–2187.

Willard MB, Hulebak KL (1977) The intra-axonal transport of polypeptide H: evidence for a fifth (very slow) group of transported proteins in the retinal ganglion cells of the rabbit. Brain Res 136:289–306.

Willard MW (1983) Techniques for studying axonally transported proteins. In: Current methods in cellular neurobiology, Vol 2 (McKelvey J, Barker J, eds), pp 35–84. New York: Wiley.